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RESEARCH PAPER

Chemically forced dormancy termination mimics natural dormancy progression in potato tuber meristems by reducing ABA content and modifying expression of genes involved in regulating ABA synthesis and metabolism*

Luis Destefano-Beltrán, Donna Knauber, Linda Huckle and Jeffrey Suttle†

United States Department of Agriculture, Agricultural Research Service, Sugarbeet and Potato Research Unit, Northern Crop Science Laboratory, PO Box 5677, State University Station, Fargo, ND 58105-5677, USA

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Abstract

The length of potato tuber dormancy depends on both the genotype and the environmental conditions during growth and storage. Abscisic acid (ABA) has been shown to play a critical role in tuber dormancy control but the mechanisms regulating ABA content during dormancy, as well as the sites of ABA synthesis, and catabolism are unknown. Recently, a temporal correlation between changes in ABA content and certain ABA biosynthetic and catabolic genes has been reported in stored field tubers during physiological dormancy progression. However, the protracted length of natural dormancy progression complicated interpretation of these data. To address this issue, in this study the synthetic dormancy-terminating agent bromoethane (BE) was used to induce rapid and highly synchronous sprouting of dormant tubers. The endogenous ABA content of tuber meristems increased 2-fold 24 h after BE treatment and then declined dramatically. By 7 d post-treatment, meristem ABA content had declined by >80%. Exogenous [^{3}H]ABA was readily metabolized by isolated meristems to phaseic and dihydrophaseic acids. BE treatment resulted in an almost 2-fold increase in the rate of ABA metabolism. A differential expression of both the *StNCED* and *StCYP707A* gene family members in meristems of BE-treated tubers is consistent with a regulatory role for *StNCED2* and the *StCYP707A1* and *StCYP707A2* genes. The present results show that the changes in ABA content observed during tuber dormancy progression are the result of a dynamic equilibrium of ABA biosynthesis and degradation that increasingly favours catabolism as dormancy progresses.

Key words: ABA, bromoethane, dormancy, potato, qRT-PCR, *Solanum tuberosum*, tuber.

Introduction

Potato tuber dormancy has been defined as the physiological state in which autonomous sprout growth will not occur, even when the tuber is placed under ideal growing conditions. (Coleman, 1987; Burton, 1989). The underlying mechanisms controlling dormancy in potato tubers are unknown. However, in the last 10 years, the role of plant hormones has been firmly established. It has been shown that both abscisic acid (ABA) and ethylene are needed for dormancy induction, but only ABA is required to maintain dormancy (Suttle and Hultstrand, 1994; Suttle, 1995, 1998*a*). Additionally, increases in cytokinin sensitivity and content appear to be principal factors leading to the loss of dormancy (Suttle, 1998*b*, 2000), whereas changes in endogenous IAA and GA content appear to be more closely related to the regulation of subsequent sprout growth. Recently, a model for hormonal regulation of tuber dormancy has been proposed (Suttle, 2004).

The amount of ABA in plant tissues is controlled by the combined rates of synthesis and catabolism. This dynamic balance is influenced by an array of develop-

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† To whom correspondence should be addressed. E-mail: suttlej@fargo.ars.usda.gov

mental and environmental signals and interplay with other phytohormones (Cutler and Krochko, 1999). In vascular plants, ABA is synthesized from a C_{40} carotenoid precursor which is converted by a series of enzymatic steps into the C_{15} phytohormone (Nambara and Marion-Poll, 2005). Genes for most of the components of this pathway have been identified and cloned from different plant species (Nambara and Marion-Poll, 2005). Also, in most plant tissues, the principal ABA catabolic pathway begins with hydroxylation of the C-8′ methyl group to form the unstable intermediate 8′-hydroxy-ABA that spontaneously rearranges to phaseic acid (PA) (Cutler and Krochko, 1999). Recently, four Arabidopsis P450 *CYP707A* genes have been identified that encode ABA 8′-hydroxylases (Kushiro *et al*., 2004; Saito *et al*., 2004). Earlier studies have demonstrated that the 8′-hydroxylase pathway leading to PA and its metabolite dihydrophaseic acid (DPA) is the predominant route of ABA degradation in potato tubers (Suttle, 1995).

The length of tuber dormancy depends on both the genotype and environmental conditions during growth and storage. In many potato cultivars, natural dormancy progression occurs over a period of many months. Tuber formation under cool and wet conditions may extend the dormant period, whereas hot and dry conditions typically shorten dormancy (Burton, 1989). Also between 3 °C and 25 °C, tuber dormancy duration is inversely related to post-harvest storage temperature, but storage temperatures below 3 °C or above 30 °C are stressful and result in premature sprouting (Wurr and Allen, 1976). In addition, exposure to a wide variety of physical conditions or chemical agents results in the rapid termination of dormancy (Coleman, 1987). Apart from acting as stressors, these treatments have little in common and their mechanisms of action are unidentified.

Meristems on different potato tubers from a single harvest can emerge from dormancy weeks apart (Burton, 1989; Suttle, 2000). Additionally, there is significant variability depending on the position of the meristem (i.e. apical versus lateral) on the tuber (Burton, 1989). To minimize both the protracted time frame of natural dormancy progression and the meristem asynchrony for each tuber, a chemically forced dormancy system using bromoethane (BE) has been adopted which compresses the natural unpredictable dormancy period from ~150 d to 10 d and induces uniform sprouting of all tuber meristems. Using this system, changes in the 5-methylcytosine content of potato tuber meristem DNA (Law and Suttle, 2002) and tuber histone H3 and H4 multi-acetylation patterns (Law and Suttle, 2004) have been characterized during natural and BE-induced dormancy progression. Also, global gene expression profiles in potato tuber meristems during natural and after BE-induced dormancy termination have been examined using the TIGR (www.tigr.org) 10K potato microarray (MA Campbell and J Suttle, unpublished results).

Previously, the effects of post-harvest storage on ABA content and metabolism, and on the expression of genes involved in key steps of the ABA biosynthetic and catabolic pathways have been reported (Destefano-Beltrán *et al*., 2006). A temporal correlation between changes in ABA content and certain biosynthetic and catabolic genes was noted. In particular, increases in ABA content were associated with increased expression of a member of the 9-*cis*-epoxycarotenoid dioxygenase gene family (*StNCED2*) while decreases in ABA content were correlated with increased expression of two *StCYP707A* genes encoding putative ABA 8'-hydroxylases. However, the extended time span required for natural dormancy progression in these tubers (~150 d) complicated interpretation of these data.

In the present study, changes in ABA content and metabolism, and the expression of selected ABA biosynthetic and catabolic genes were determined in meristems isolated from BE-treated tubers. Present findings confirm that changes in ABA content observed during tuber dormancy progression are the result of a dynamic equilibrium between ABA biosynthesis and degradation that increasingly favours catabolism as dormancy progresses.

Materials and methods

Plant material and BE treatment

Experiments were conducted using *Solanum tuberosum* L. cv. Russet Burbank tubers harvested in late September in north-western Minnesota. Prior to storage, the tubers were conditioned at 20 °C for 2 weeks to permit wound-healing. Standard storage conditions were 3 °C and >95% RH in the dark. Tubers were transferred to room temperature and stored in the dark 3 d prior to use. Bromoethane (BE) treatments were performed as described by Law and Suttle (2002). Briefly, tubers were placed in sealed Plexiglas chambers and were exposed to 0.2 ml BE per litre of container volume for 24 h at room temperature in the dark. After treatment, tubers were placed in a chemical hood for 6 h at room temperature (to allow the release of absorbed BE vapours) and subsequently stored at 20 °C in the dark for up to 10 d. Using a 1 mm curette, with the aid of a dissecting microscope, meristems were excised prior to treatment (day 0), immediately after treatment (day 1), and at various times thereafter. Excised meristems were immediately frozen in liquid nitrogen and stored at −80 °C for later use (ABA quantification and qRT-PCR studies).

All experiments described in this report were conducted a minimum of two times with qualitatively similar results. Each sample within an experiment was replicated at least three times. Results from typical experiments are presented.

De novo *DNA synthesis*

DNA synthesis in control and BE-treated meristems was assayed by determining the rate of [^{3}H]thymidine incorporation into TCA-insoluble material. Groups of 10 meristems were incubated in 1 ml of uptake buffer (10 mM MES/KOH, pH 5.7, 50 μg $^{-1}$ ml chloramphenicol) containing 185 kBq [methyl,1′,2′,-^{3}H]thymidine (4.66 TBq mmol^{-1}; Amersham) with shaking at room temperature. After 4 h, meristems were rinsed 3× with deionized water, once with 5 mM unlabelled thymidine, blotted dry, frozen in liquid nitrogen,

and stored at −80 °C. Tissue extraction and determination of percentage radiolabel incorporation into TCA-precipitable material were conducted as previously reported (Law and Suttle, 2004). Values presented are means ±standard error (n=3).

ABA extraction

Frozen tissue samples (100 meristems per replicate) were allowed to thaw at 4 °C in 80% (v/v) aqueous acetone. After mechanical homogenization (4 °C), the extract was clarified by centrifugation (10 000 g for 15 min). The resulting pellet was re-extracted in 80% (v/v) aqueous acetone and, after standing for ⩾2 h (4 °C), was recentrifuged and the supernatants combined. Depending on the sample, a total of 50–100 ng $^2H_6$(+)-ABA (OlChemIm Ltd., Olomouc, Czech Republic) was added as an internal standard. The supernatants were dried under a stream of N_2 (40 °C) and redissolved in 5 ml 1 M formic acid. The acidified extracts were loaded onto a 150 mg MCX Oasis cartridge (Waters Associates, Milford, MA, USA). The cartridge was then washed with 5 ml 1 M formic acid, followed by 5 ml methanol. The methanol fraction was collected and taken to dryness under a stream of N_2 (40 °C) before being redissolved in 10 mM ammonium acetate (pH 5.6).

ABA quantification

ABA was quantified by high performance liquid chromatography–mass spectrometry (HPLC–MS) using a Thermo Electron Surveyor MSQ system and a 2.1 mm ×150 mm 5 µm Hypersil Gold Column (Thermo-Finnigan, San Jose, CA, USA). HPLC solvents were: (A) 10 mM ammonium acetate (pH 5.6) and (B) methanol (0.2 ml min^{-1}). Starting conditions were 40% B, a linear gradient to 80% B in 5 min followed by a linear gradient to 100% B in 5 min. Detection and quantification were performed using the MSQ operating in the negative ion, electrospray ionization mode with a probe temperature of 460 °C, cone voltage of 30 V, and needle voltage of 4.5 kV. Ions (m/z) monitored were: 263 for ABA and 269 for $[^2H]_6$ABA. Each sample replicate was injected at least twice.

ABA metabolism in BE-treated meristems

Fresh meristems were isolated with a curette and briefly washed in deionized water followed by incubation buffer (10 mM MES-KOH, pH 5.7) at room temperature. Groups of 10 meristems were then incubated in 1 ml of buffer containing 37 kBq [^{3}H](±)-ABA (550 GBq $mmol^{-1}$; American Radiochemicals Inc., St Louis, MO, USA) at room temperature with constant agitation. After 4 h, the meristems were removed, washed extensively with running deionized water, blotted dry, frozen in liquid N_2, and stored at −80 °C. Meristems were mechanically homogenized in 80% (v/v) aqueous acetone (4 °C) and the extracts clarified by centrifugation (10 000 g for 15 min). The supernatants were taken to dryness under a stream of nitrogen (40 °C) and redissolved in 1% (v/v) acetic acid. Extracts were fractionated by reverse-phase HPLC coupled with an in-line radioactivity monitor as described previously (Suttle, 1995). Metabolite identification was achieved by co-chromatography with authentic standards (Suttle, 1995).

RNA extraction

Total RNA was isolated from meristem samples (100 eyes each) using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. RNA quality was determined by agarose gel electrophoresis in 1× TBE followed by ethidium bromide staining and UV light visualization. Before analysing for specific mRNAs, total RNA was treated with DNA-*free*™ (Ambion) to eliminate genomic DNA contamination.

qRT-PCR analysis

Total RNA (1.5 µg) was reverse transcribed using the RETROscript® kit (Ambion), with oligo dT_{18} as the primer, according to the manufacturer's recommendations. The cDNA was then diluted in a total volume of 170 µl with sterile RNase-free water. Amplification of specific regions of targeted genes and real-time detection of amplicon production was conducted using a DNA Engine Opticon™ 2 (Bio-Rad, Hercules, CA, USA). Target genes and their GenBank accession numbers were: *StZEP*, DQ206629; *StNCED1*, AY662342; *StNCED2*, AY662343; *StABA2*, AY662344; *StAAO3*, DQ206634; *StCYP707A1*, DQ206630; *StCYP707A2*, DQ206631; and *StCYP707A3*, DQ206632. The sequences of the primer pairs used for each gene are presented in Table 1. PCR reactions were carried out under the following conditions: 94 °C/2 min (one cycle); 58 °C/1 min (one cycle); 72 °C/1 min (one cycle); 94 °C/30 s, 58 °C/30 s; 72 °C/45 s (35 cycles).

PCR amplification of a single product of the correct size for each gene was confirmed by agarose gel electrophoresis and double-strand sequencing. The amplified fragment of each gene was subcloned and used to generate efficiency curves. Relative fold expression for each gene was calculated by the method of Pfaffl (2001). The transcript of *StCCD* (GenBank accession number DQ206633), a possible orthologue of *AtCDD4*, was used to standardize each reaction run with respect to RNA integrity, sample loading, and inter-PCR variations since it exhibited minimal changes in expression in meristems over the time frame examined (data not presented).

Results

Tubers were stored at 3 °C in the dark (95% RH) immediately after skin setting and wound healing (2 weeks after harvest). Under these storage conditions, tubers were completely dormant (defined as no sprout growth after

Table 1. *Forward and reverse primers used for qRT-PCR expression analyses of ABA metabolic genes and estimated absolute copy transcript copy number in tuber meristems prior to BE treatment*

Gene	Forward primer	Reverse primer	Abundance (copies/µg total RNA)[a]
StZEP	TATCTGAGAAAGCAAATGACC	GTAGGGAAGTTTGGAGACGT	$1.28\pm0.17\times10^7$
StNCED1	GGAAATCAACAAGAAAAGCCA	ATATTTGTTGTCACCATAAATGAA	$1.51\pm0.39\times10^6$
StNCED2	GGGACTTTCATTAGCTCAAAGGACTTGC	GCGATGTAAATTTGAATTACTATTATTCGCTCA	$3.66\pm0.40\times10^7$
StCYP707A1	GTACAGGTGGTCTATGGTGG	CGATGCTTGTTGTCTTGATGTTGATTG	$1.26\pm0.03\times10^4$
StCYP707A2	TGAGTGTGTCAAGGCTGAACAGAAAGTT	GAATGCCACTACCAGATCCTACCACTTC	$1.65\pm0.06\times10^6$
StCYP707A3	TGTGTTTAAGCTCTTTGATTGTGGTG	TGCTCGAAGAACTAGCTTTCTTAGC	$3.45\pm0.48\times10^5$
StCCD	GAGGATGGACATGATACATGC	GACATGAGCCACAATGTCAAG	$7.93\pm1.63\times10^5$

[a] Mean ±standard error (n=3).

2 weeks at 20 °C) for at least 48 d of storage. As expected, dormancy weakened after that and by 97 d tubers presented limited sprout growth (<5 mm). After 125 d of storage, tubers were completely non-dormant and exhibited rapid sprout growth (5–15 mm) after transfer to 20 °C. The tubers used in this study had been stored for ~40 d and were completely dormant at the time of BE treatment.

Effects of BE on tuber meristem reactivation and growth

BE treatment rapidly terminates tuber dormancy in Russet Burbank potatoes (Coleman, 1983; Law and Suttle, 2004). Because the timing and magnitude of BE action in tubers varies from year to year in an unpredictable manner, it was necessary to determine the efficacy and timing of BE-induced dormancy termination in each lot of tubers used. Twenty-four hours after the start of BE treatment (all time points refer to time from the start of BE treatment), incorporation of [^{3}H]thymidine was reduced by more than half but returned to initial values 3–4 d post-treatment (Table 2). By 7 d, thymidine incorporation in BE-treated meristems was nearly twice that of controls and remained elevated after 9 d. Visible sprout growth in BE-treated tubers could be detected (i.e. ≤1 mm) after 6–7 d and by 9 d was ≥2 mm. BE induced simultaneous sprouting of nearly all eyes on treated tubers, thereby minimizing the spatial disparity in sprouting rates often observed between eyes in the apical and basal portions of tubers.

Changes in ABA homeostasis in tuber meristems following BE treatment

The rapid termination of tuber meristem dormancy following BE treatment was accompanied by wholesale changes in ABA content. The ABA content of tuber meristems increased 2-fold in the first day after BE treatment and then declined 4-fold from days 1 to 4 post-treatment, remaining constant thereafter (Fig. 1). The marked decline in ABA content between days 1 and 4 suggested an increased rate of ABA metabolism. To study this further and assess the catabolic capacity of meristems following BE treatment, the metabolism of exogenous [^{3}H]ABA was examined (Fig. 2). Isolated meristems readily metabolized exogenous ABA to PA and DPA. Only limited amounts of ABA–glucose ester and an unknown minor metabolite thought to be a conjugate of DPA were formed. Prior to BE treatment, 80% of the recovered radioactivity was associated with ABA with the remainder equally distributed between PA and DPA. The rate of metabolism was unchanged at 24 h but, by 4 d post-treatment, began to rise primarily as a result of an increase in PA formation. The rate of ABA metabolism continued to increase and, by day 7, the rate of PA formation had increased 2-fold over initial values. These results demonstrated that, following BE treatment, the capacity of meristems to metabolize ABA via the 8'-hydroxylase-catalysed pathway increased and suggested that this increase was at least partially responsible for the decline in ABA content between 1 d and 4 d post-treatment.

Table 2. *Effects of BE treatment on DNA synthesis and sprout growth in potato tuber meristems*

Intact tubers were treated with BE for 24 h. Meristems were excised from control and BE-treated tubers at the indicated times, exposed to [^{3}H]thymidine for 4 h, and the amount of TCA-insoluble radioactivity was determined. Sprout growth was assessed visually.

Days after start of treatment	[^{3}H]Thymidine incorporation (% taken up)[a]		Sprouting[b]
	Control	BE treated	
1	2.44±0.70	1.08±0.27**	0
3	2.13±0.53	2.02±0.65	0
4	2.12±0.46	2.06±0.81	0
6	3.12±0.78	4.18±0.96*	+/−
7	2.55±0.65	4.88±1.03**	+
9	2.04±0.48	4.87±1.25**	+++

[a] * and ** indicate difference from control value at P=0.1 and P=0.05, respectively.

[b] 0, no visible sprouts; +/−, sprouts ≤1 mm; +, sprouts 1–2 mm; +++, sprouts 2–5 mm.

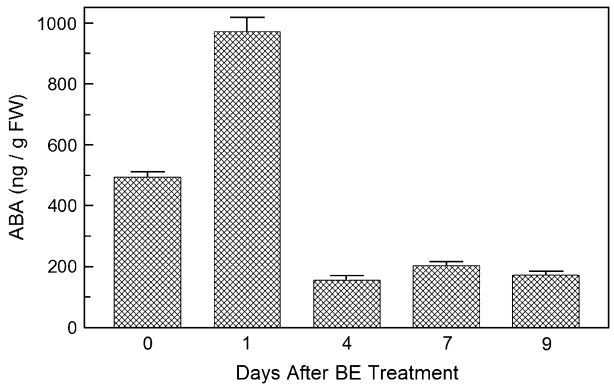

Fig. 1. Effects of BE treatment on endogenous content of free ABA in meristems isolated from field-grown Russet Burbank tubers (storage season 2004–2005). Dormant tubers were treated with BE for 24 h and, at the indicated times, meristems from treated tubers were excised, frozen in liquid nitrogen, and stored at −80 °C. ABA was quantified by HPLC-MS as described in the Materials and methods. Points are means and bars denote standard error (n=3).

Expression of ABA biosynthetic and catabolic machinery during chemically forced dormancy exit

To examine the expression of ABA biosynthetic and catabolic genes during potato tuber dormancy, real time PCR was performed on RNA isolated from meristems from BE-treated Russet Burbank tubers. For purposes of comparison, individual gene expression levels on day 0 were set to one. Using un-normalized control data (in triplicate) and experimentally determined amplification efficiency curves for each gene, the approximate number of transcripts (per microgram of total RNA) in the meristems of tubers at day 0 was calculated and is summarized in Table 1. As expected, transcript copy number varied considerably among genes.

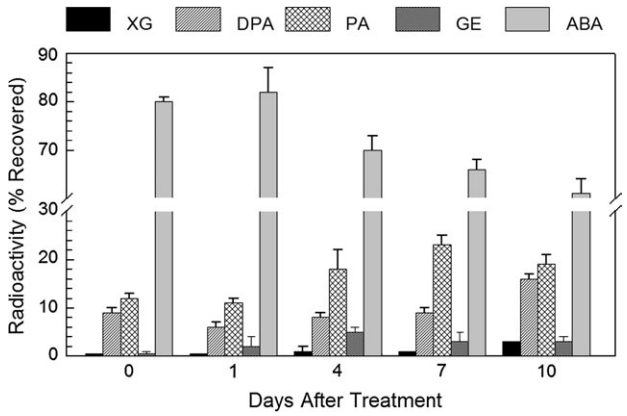

Fig. 2. Effects of BE treatment on metabolism of [^{3}H](±)-ABA in isolated tuber meristems. Meristems were isolated from tubers at various times after BE treatment and were incubated on solutions of [^{3}H](±)-ABA in buffer for 4 h. Distribution of radiolabelled metabolites was determined by HPLC using an inline radioactivity monitor as described in the Materials and methods. Points are means and bars denote standard error (n=3).

Transcript abundance of *StCYP707A1* was considerably lower than those of the other genes. Interestingly, the copy number for *StNCED2* was ~20-fold higher than that for *StNCED1*.

The relative expression of key ABA biosynthetic genes in meristems after BE treatment is shown in Fig. 3. Transcript abundances of genes encoding the initial steps of ABA biosynthesis (*StZEP* and *StNCED2*) increased ~5- and ~15-fold, respectively, 1 d after BE treatment and fell to initial levels thereafter. Surprisingly, *StNCED1* expression was down-regulated throughout the study.

The relative expression of three potato *CYP707A* genes in tuber meristems after chemically forced dormancy break is presented in Fig. 4. In general, each gene showed a unique expression pattern. Thus, *StCYP707A1* expression increased dramatically (>6-fold) during day 1 and remained significantly elevated for the duration of the study. By contrast, *StCYP707A2* expression showed no increase during the first 24 h, increased slightly (but significantly) between 1 d and 4 d after BE treatment, and increased >2-fold from day 4 to day 7 before levelling off between 7 d and 9 d. Finally, *StCYP707A3* gene expression showed a sharp transient rise of ~6-fold during day 1, followed by an abrupt decline to basal levels by day 7 before increasing again ~4-fold from day 7 to day 9.

Discussion

From a developmental perspective, potato tubers are highly adapted and compressed underground stems, whose major functions include the import and storage of assimilates and the seasonal regeneration of the above-ground plant. The life cycle of this organ, from tuber induction to sprouting, is governed by a complex interacting set of regulatory cues that control both the functional switch from sink to source organ

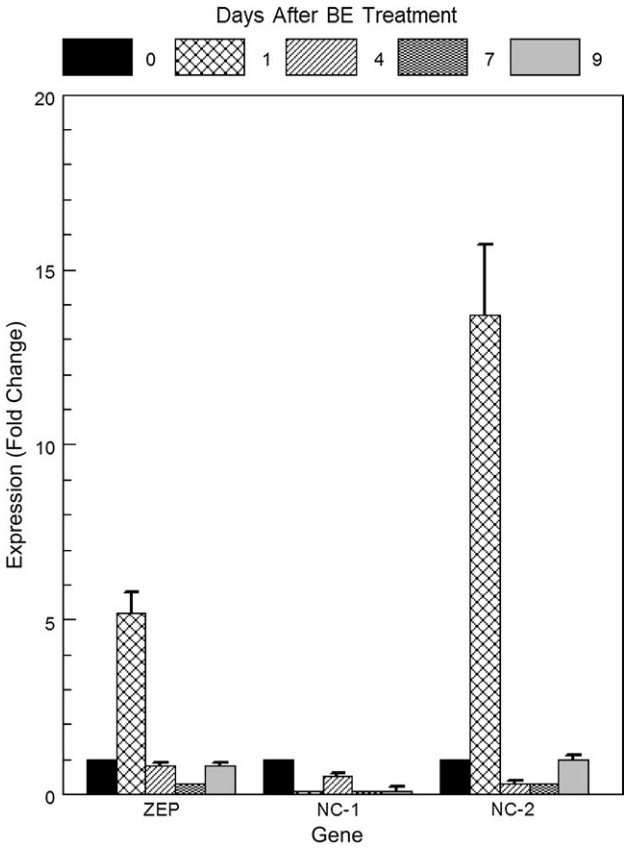

Fig. 3. Quantitative expression analysis of key ABA biosynthetic genes in tuber meristems after BE treatment. At the indicated times, meristems from untreated (day 0) and treated tubers were isolated, frozen in liquid nitrogen, and stored at −80 °C. Real time RT-PCR was performed on total RNA isolated from untreated and treated meristems. Relative fold expression for each gene was calculated by the method of Pfaffl (2001) using *StCCD* as the reference gene. Expression for each gene is presented as fold change relative to untreated controls harvested immediately prior to the start of BE treatment (day 0). Each gene was analysed in triplicate. Values presented are means ±standard error (n=3). ZEP, Zeaxanthin epoxidase; NC-1, 9-*cis*-epoxycarotenoid dioxygenase-1; NC-2, 9-*cis*-epoxycarotenoid dioxygenase-2.

and the concurrent stop–start growth cycle of tuber meristems (Jackson, 1999; Faivre-Rampant *et al*., 2004*a*, *b*).

An important commercial feature of the potato tuber life cycle is the length of dormancy; the indeterminate period after harvest in which meristem growth ceases. Tuber dormancy is influenced in a complex manner by both genetics and environment with both most likely mediated by changes in hormone status (Suttle, 2004; Vreugdenhil, 2004). In fact, sustained synthesis and action of endogenous ABA is required for both the initiation and maintenance of tuber dormancy (Suttle and Hultstrand, 1994). Although, the fundamental molecular mechanisms controlling ABA content during dormancy as well as the sites of ABA synthesis and catabolism in tubers are unknown, an intriguing and complex picture has started to emerge.

Recently, changes in ABA content and expression of genes encoding key enzymes of the ABA biosynthetic

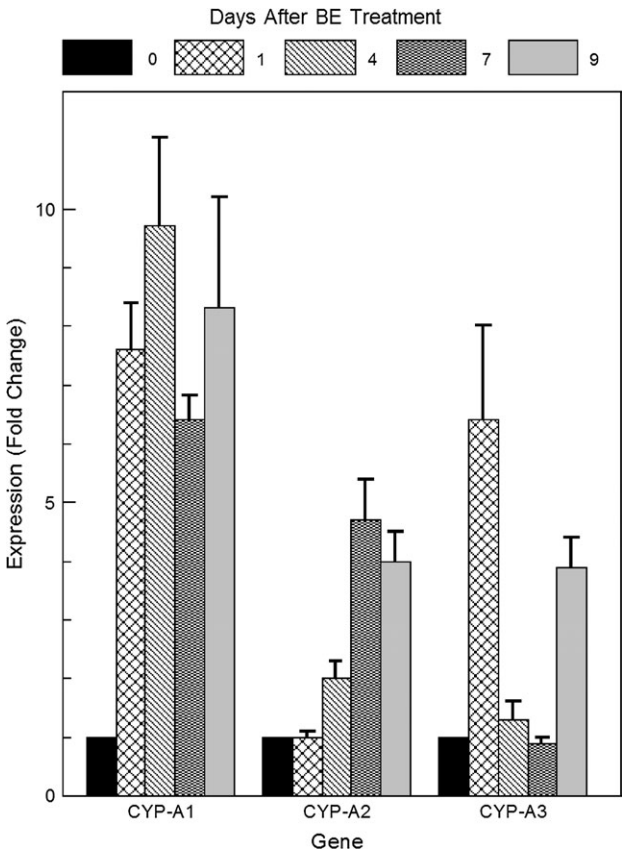

Fig. 4. Quantitative expression analysis of *StCYP707A* genes in tuber meristems after BE treatment. At the indicated times meristems from untreated (day 0) and treated tubers were isolated, frozen in liquid nitrogen, and stored at −80 °C. Other details were as described in the legend to Fig. 3.

pathway and three putative ABA 8′-hydroxylases thought to initiate ABA catabolism have been reported for three different tuber tissues (meristems, surrounding periderm, and underlying cortex) during natural dormancy progression of cold-stored tubers (Destefano-Beltrán *et al*., 2006). Natural release from dormancy is not uniform and meristems on different potato tubers from a single harvest can emerge from dormancy weeks apart (Burton, 1989; Suttle, 2000). This asynchrony together with the extended time span required for natural dormancy progression complicated interpretation of these data. To minimize these limitations, in the present study, analyses have been extended to dormant tubers chemically forced to break dormancy by BE treatment.

The ability of BE to terminate potato tuber dormancy effectively was first reported by McCallum (1909) and subsequently confirmed by Denny (1926). BE is used in both potato disease certification programmes and the potato seed industry to stimulate early sprouting and emergence (Coleman, 1983). Despite its efficacy in terminating tuber dormancy, the mechanisms-of-action of BE in this regard are unknown. Using BE, the protracted duration of the natural dormancy cycle has been compressed from ~150 d to a much more predictable and uniform 10 d period. BE treatment of dormant Russet Burbank potato tubers resulted in visible (>2 mm) sprout growth from nearly all eyes after 7–9 d, which is preceded by increased *de novo* DNA synthesis as suggested by the [^{3}H]thymidine incorporation assays (Table 2). BE treatment has previously been used to monitor changes in chromatin remodelling (histone H3 and H4 multi-acetylation, DNA cytosine methylation) during tuber dormancy progression (Law and Suttle, 2002, 2004). These studies demonstrated that a chemically forced dormancy break mimics natural dormancy release in at least these two important developmentally related aspects in potato tubers.

ABA is actively metabolized in tuber tissues throughout dormancy and, in general, tuber ABA content has been shown to decline during storage (Suttle, 1995; Biemelt *et al*., 2000). In the present study, it was shown that following BE treatment, ABA content in meristems increased by ~2-fold after 24 h, had declined dramatically (>80%) by day 4, and then remained at levels >2-fold lower than those at the start (0 h). This pattern of change in ABA content is reminiscent of that observed in tuber meristems during natural dormancy progression where ABA content rose 43% during the first 27 d of cold-storage and then fell steadily towards the end of the study (~150 d), except for a small but statistically significant increase at 111 d of cold-storage (Destefano-Beltrán *et al*., 2006). Also, the present results are in general agreement with earlier reports describing changes in ABA content in whole potato tubers undergoing natural dormancy progression (Korableva *et al*., 1980; Coleman and King, 1984; Cvikrová *et al*., 1994; Biemelt *et al*., 2000) or following release from dormancy by synthetic cytokinin or heat stress (Ji and Wang, 1988; van den Berg *et al*., 1991).

Previous studies demonstrated that the decline in endogenous ABA content during tuber dormancy was not accompanied by a commensurate increase in ABA–glucose ester and that exogenous [^{3}H](+)-ABA was metabolized in intact tubers exclusively to PA and ultimately DPA (Suttle, 1995). Sorce *et al*. (1996) also found an increase in tuber DPA content during storage, while ABA glucose ester content fell during this time period. Collectively, these results suggest that, during tuber dormancy, ABA catabolism occurred predominately via oxidation initially catalysed by cytochrome P450 ABA 8′-hydroxylase (Cutler and Krochko, 1999). The metabolism studies described in the present study with meristems of BE-treated tubers not only confirmed that ABA 8′-hydroxylation was the main route for ABA metabolism but also demonstrated that dormancy break was preceded and accompanied by an increased rate of ABA degradation. A similar increase in ABA catabolism was observed in meristems during natural dormancy progression (Destefano-Beltrán *et al*., 2006).

In tuber meristems, a temporal correlation between changes in ABA content and expression of certain ABA biosynthetic and catabolic genes has recently been determined during natural dormancy progression (Destefano-Beltrán

et al., 2006). In particular, changes in ABA content closely mirrored the expression of a member of the 9-*cis*-epoxycarotenoid dioxygenase gene family (*StNCED2*). The role, if any, of the other NCED member (*StNCED1*) was uncertain because its expression pattern was unrelated to changes in ABA content. Furthermore, *StNCED2* transcript abundance was 32-fold higher than that of *StNCED1*. Likewise, decreases in ABA content correlated with up-regulated expression of two members of the potato *CYP707A* gene family (*StCYP707A1* and *StCYP707A2*) that encode putative ABA 8'-hydroxylases. Expression of a third member (*StCYP707A3*) did not change significantly during dormancy. Consequently, in the present work, it was also decided to monitor the expression of these genes in tuber meristems after BE treatment with the purpose of verifying their pattern of expression and, in the case of the *NCED* family, resolving the uncertainty of the role of *StNCED1*.

In many tissues, NCED is considered to be the regulatory enzyme of ABA biosynthesis and its expression is well correlated with endogenous ABA content (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005). Also in most plant tissues examined, NCED is encoded by a gene family (Chernys and Zeevaart, 2000). In potato tubers, two *NCED* genes (*StNCED1* and *StNCED2*) have been characterized (Table 1). In meristems of BE-treated tubers, *StNCED2* transcript abundance not only exceeded that of *StNCED1* by 24-fold (Table 1) but its expression closely mirrored changes in ABA content, while *StNCED1* transcript showed little variation (cf. Figs 1, 3). Moreover, in other studies, the increase in ABA content upon wounding in tuber cortical tissues was accompanied by a parallel increase in *StNCED1* (but not *StNCED2*) transcript abundance (L Destefano-Beltran *et al*., unpublished data). These data support the earlier suggestion that ABA biosynthesis in meristems during dormancy progression is mediated and perhaps controlled by the *StNCED2*-encoded protein. Differential *NCED* expression has also been described in both avocado and *Arabidopsis*, where multiple *NCED* genes have been identified that exhibit either tissue-specific or developmentally regulated expression (Chernys and Zeevaart, 2000; Tan *et al*., 2003). Despite several attempts using degenerate primers, only two *NCED* genes were identified in potato tuber cDNA. Nevertheless, the presence of additional family members in potato tissues cannot be discounted. Interestingly, the TIGR tomato gene index with a collection of ~32 000 unique sequences contains only one *NCED* member.

The marked (>80%) decline in endogenous meristem ABA content between 1 d and 4 d after BE treatment (Fig. 1) suggested an increased rate of ABA catabolism during this period. Consistent with this notion, the metabolism of exogenous [^{3}H]ABA remained constant for 24 h after treatment and increased substantially thereafter (Fig. 2). By 7 d post-treatment, metabolism of exogenous ABA had increased >2-fold over the initial levels. Moreover, analysis of the metabolites formed indicates that ABA oxidation (not esterification) was the predominant route of catabolism in these tissues. These data are consistent with previous metabolism studies using whole tubers (Suttle, 1995).

Expression analysis of three members of the potato *CYP707A* gene family following BE treatment supported this hypothesis. Expression of the least abundant member of this gene family (*StCYP707A1*) increased >7-fold during day 1 and remained elevated thereafter, whereas expression of the most abundant family member (*StCYP707A2*) doubled between days 1 and 4 and doubled again between days 4 and 7 concurrent with the observed increase in ABA metabolism. It is important to note that the difference in transcript abundance between these two genes was ~130-fold. By contrast with previous results for tubers naturally exiting dormancy, expression of *StCYP707A3* in BE-treated tubers rose ~7-fold during day 1 but returned to initial levels by day 4. This discrepancy could be explained by other results (L Destefano-Beltran *et al*., unpublished data) in which *StCYP707A3* expression was transiently increased in the tuber cortex by wounding. As such, the transient increase in *CYP707A3* expression 24 h after BE treatment may be stress-mediated and unrelated to dormancy exit.

Interestingly, expression of *StZEP* was strongly (>5-fold) up-regulated 24 h after BE treatment (Fig. 3). In most plant tissues including potato tubers, ZEP (zeaxanthin epoxidase) is not a rate-limiting enzyme in ABA biosynthesis (Römer *et al*., 2002). However, endogenous carotenoid levels in white-fleshed potatoes such as Russet Burbank are quite low (Morris *et al*., 2004) and the >2-fold increase in ABA formation 1 d after BE treatment may have depleted certain of these pools (notably *cis*-violoxanthin and *cis*-neoxanthin), thereby stimulating net carotenogenesis.

Collectively, the present results suggest that the initial increase in ABA content following BE treatment was a result of increased expression and activity of *StNCED2* together with basal ABA 8′-hydroxylase activity. Further, the dramatic decline in ABA content between days 1 and 4 may reflect decreased expression of *StNCED2* coupled with dramatically increased *StCYP707A* expression and activity. From the data presented, it is unclear which of the *StCYP707A* genes examined is primarily responsible for the increased ABA turnover in meristems following BE treatment. Despite sustained elevations in the expression of all three *StCYP707A* genes after day 4 (Fig. 4), ABA content remained nearly constant, indicating that ABA synthesis and catabolism were in near-equilibrium. It is possible that additional ABA was being formed by accelerated release from a conjugate such as ABA–glucose ester. Alternatively, ABA-8′-hydroxylase activity in tubers may also be regulated post-transcriptionally. Further studies are needed to clarify this issue.

In tubers, as in other plant tissues (Chernys and Zeevaart, 2000; Tan *et al*., 2003; Kushiro *et al*., 2004), multiple genes

encode these potentially regulatory steps. Thus, unequivocal identification of specific genes regulating ABA homeostasis in potato tubers during dormancy will require additional experimental evidence. Current research using both antisense and RNAi strategies is directed toward elucidating the role(s) of individual gene members in these two families in the regulation of ABA levels during tuber dormancy and post-harvest storage.

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